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Design and synthesis of ^{99m}Tc-citro-folate for use as a tumor-targeted radiopharmaceutical

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ABSTRACT

Folate conjugates exhibit high affinity for folate receptor (FR) positive cells and tissues, such as those in tumors, making them attractive candidates and of interest in diagnostic tumor imaging. The aim of study was to synthesize a novel radiopharmaceutical based folate conjugate, ^{99m}Tc-citro-folate, and to evaluate its efficiency as a targeted agent for imaging tumors that over express FR. TLC, HPLC ^{1}H NMR and LC–MS/MS methods were used to check and confirm the synthesized citro-folate. Citro-folate was labeled with Tc-99m with high labeling efficiency (97 ± 1.0%). Biodistribution of the radiolabeled conjugate ^{99m}Tc-citro-folate was investigated in vivo using two groups of rats: FR saturated and unsaturated. These experiments showed high uptake of ^{99m}Tc-citro-folate in FR rich tissues and demonstrated its sensitivity and specificity in imaging ovary and uterus. Based on the demonstrated good radiolabeling and biodistribution properties, the compound ^{99m}Tc-citro-folate may potentially be used as a radiopharmaceutical agent for imaging the FR-positive tumors.

1. Introduction

Folate receptor (FR) frequently is overexpressed in a wide range of tumor types, thereby making it an attractive target for tumor-selective radioimaging. It has specifically been identified as a biomarker for detecting ovarian carcinomas and a number of folate-based radiopharmaceuticals have been developed and evaluated for this purpose (Kim et al., 2007; Müler et al., 2006, 2008; Sudimack and Lee, 2000; Siegel et al., 2003; Guo et al., 1999; Wang et al., 1997). Past research efforts resulted in many radiolabeled folate conjugates for FR-mediated diagnostic imaging of other tumors (Kim et al., 2007; Müler et al., 2006, 2008; Sudimack and Lee, 2000; Siegel et al., 2003; Guo et al., 1999; Wang et al., 1997; Okarvi and Jammaz, 2006; Ke et al., 2003, 2004): ^{99m}Tc-PEG-folate, ^{67}Ga-DF-folate, ^{111}In-DTPA-folate, and ^{99m}Tc-HYNIC-folate. But, so far, the potential of using citro-folate (short for citric acid conjugated to folate) as a chelating agent for tumor imaging is still remain unknown. A folate-based radiopharmaceutical is synthesized at low molecular weight. A low weight compound is easily uptaken into the cell by the process of receptor-mediated endocytosis. This makes the compound more favorable in terms of its pharmacokinetic properties as compared to much larger radiolabeled antibody (Jurisson and Lydon, 1999). In this study, because of its low molecular weight and also its clinically demonstrated capability to be labeled with radioisotope Technetium 99m (^{99m}Tc), we explored the benefits of using citric acid as a ligand to folate (Ercan et al., 1992; Sager et al., 1977). ^{99m}Tc is commonly chosen for imaging in diagnostic nuclear medicine due to it is ideal energy ($E\gamma$ = 140 keV), low radiation dose, long half-life of 6 h, and wide commercial availability (Baum, 1975; Miguel et al., 2006; Subramanian et al., 1975). In the following, we describe the design and synthesis of the radiopharmaceutical ^{99m}Tc-citro-folate, and biodistribution studies aimed at evaluating its potential use for FR-targeted tumor imaging agent in nuclear medicine using in vitro and in vivo experiments.

2. Materials and methods

2.1. Materials

Folic acid, dicyclohexylcarbodiimide and hydrazine hydrate were purchased from a supplier (Sigma–Aldrich Chemical Co., Steinheim am Albuch, Germany). Citric acid and N-hydroxysuccinimide were purchased from another supplier (Merck Co., Darmstadt, Germany).

2.2. Synthesis of folate-hydrazide

First, N-hydroxysuccinimide (NHS) ester of folic acid was synthesized (Guo et al., 1999; Okarvi and Jammaz, 2006). 1 g (2.3 mmol) of folic acid was dissolved in 50 mL dimethylsulfoxide (DMSO). A 1:1 ratio molar excess of NHS and dicyclohexyl carbodiimide (DCC)

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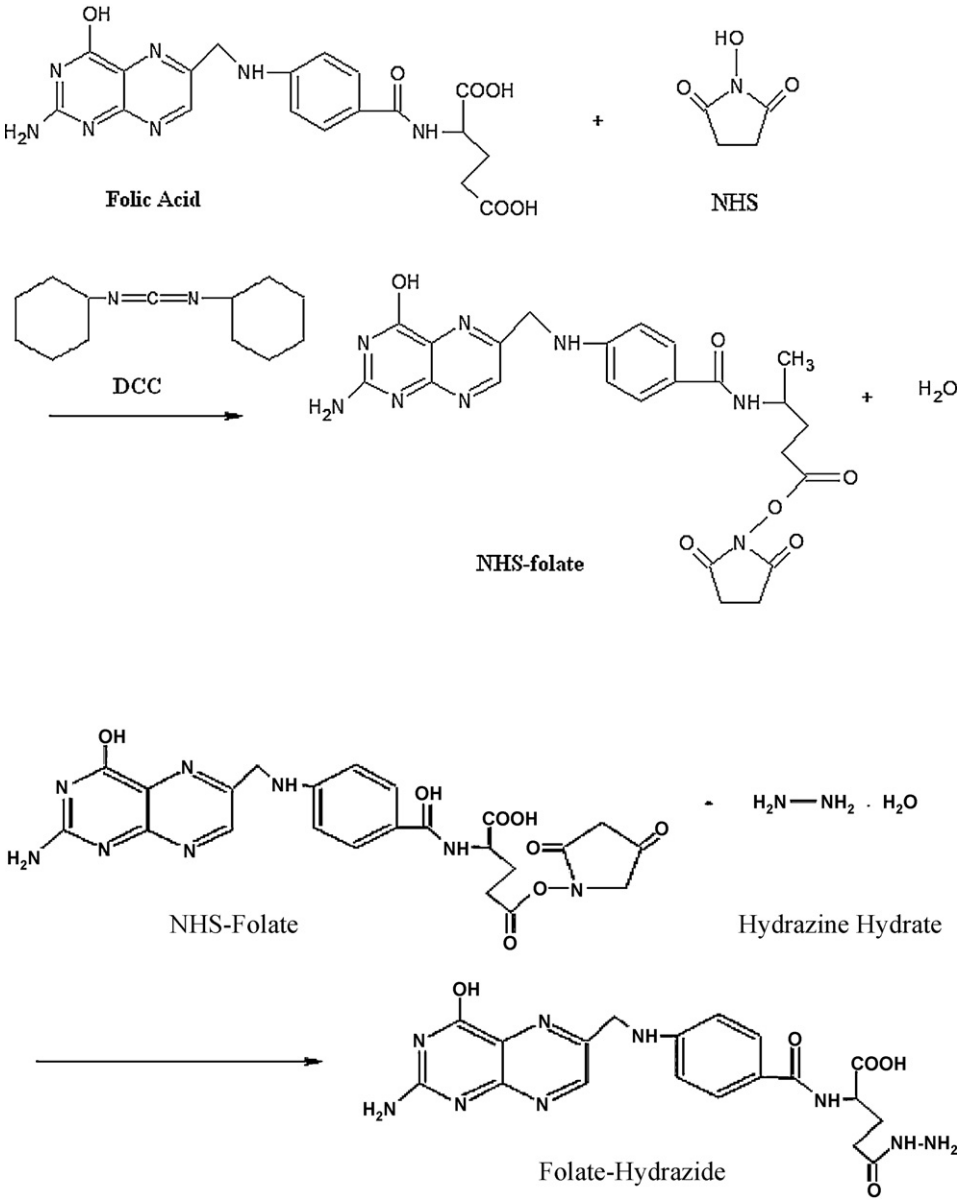

Fig. 1. Synthesis of folate-hydrazide.

were then added to the solution. The reaction was allowed to proceed for 16 h at room temperature under stirring and shielding from light. The by-product dicyclohexylurea was removed by filtration. The DMSO solution of the NHS-folate was stored at −20 °C until it was used. Then, to synthesize folate-hydrazide (Fig. 1), NHS-folate solution was slowly added to hydrazine hydrate (2 mL) under stirring at room temperature. The product folate-hydrazide was converted into hydrochloride salt by adding HCl and then precipitated with acetonitrile/diethylether 1:1. The precipitate was centrifuged, redissolved in 1 mL water and then reprecipitated with ethanol. The final pellet was washed with solvent mixtures of ethanol:diethyl ether (the ratio in mixture 1: 50/50, mixture 2: 80/20 and 100% ethanol) and then lyophilized into a yellow powder under vacuum. Then, the yellow powder, folate-hydrazide, was stored at −20 °C.

2.3. Synthesis of citro-folate

Citro-folate was synthesized as follows. Citric acid, of amount 0.0192 g (0.1 mmol), was dissolved in DMSO, then 0.0045 g (0.01 mmol) folate-hydrazide was added to prepare citric acid solution. The final solution was added 0.0056 g (0.05 mmol) $CaCl_2$ and stirred for approximately 1 h until the reaction was complete (Gourrierec et al., 2008). The resulting pellet was then washed three times by dissolution in a small volume of water followed by precipitation with ethanol. The pellet was lyophilized and stored at −20 °C (Fig. 2).

The molecular structures of NHS-folate, folate-hydrazide and citro-folate were confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR: Bruker 400 MHz spectrometer, Berlin, Germany) and liquid chromatography–mass–mass spectroscopy (LC–MS/MS: Agilent LC-MSD SL ion trap (Agilent HPLC 1100 binary pump, degasser, autosampler, column oven).

2.4. Radiochemical synthesis of ^{99m}Tc-citrate

The following procedure was used for radiolabeling. First, 0.01 g (0.05 mmol) citric acid (Brothers Chem. Co., USA) was dissolved in 2 mL water in a glass vial. The pH was adjusted to 5 with 0.5 N NaOH; 200 μL $SnCl_2$ $2H_2O$ aq. solution (1 mg/mL) was added

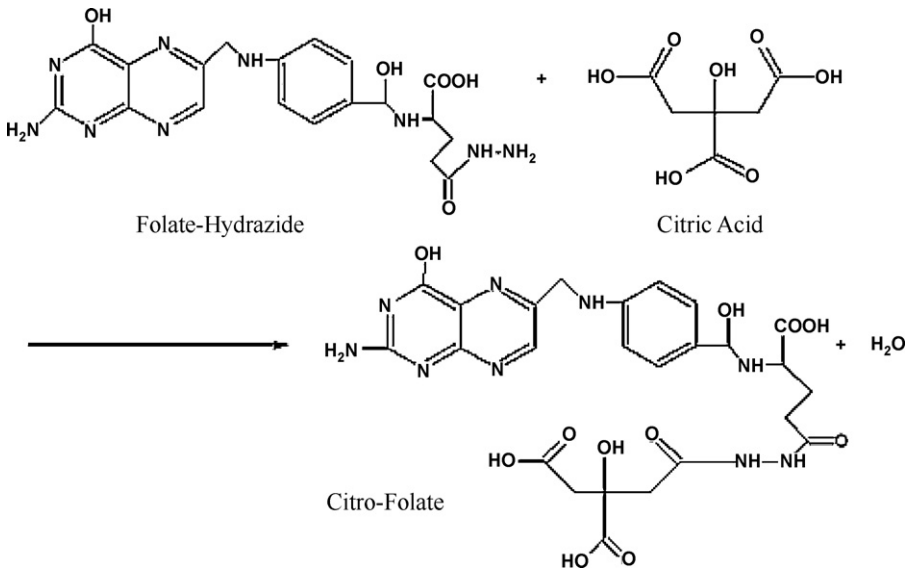

Fig. 2. Synthesis of citro-folate.

and mixed well. Next, 2 mL generator (Amersham International Inc., Amersham, UK) eluate containing 370–555 MBq of ^{99m}Tc as pertechnetate was added and left to react at room temperature for 10 min. The labeling efficiency of ^{99m}Tc-citrate was determined by Radio-Thin-Layer-Chromatography (RTLC), using ready plates of ITLC-SG and Radio High Performance Liquid Chromatography (RHPLC) using HPLC (Shimadzu, Japan). After developing in one of the three different solvent systems (0.9% sodium chloride solution and acetone and ACD [citrate-dextrose buffer solution (Eczacibasi-Baxter, Turkey)], the strips were scanned on the TLC scanner (Bioscan AR-2000, Washington DC, USA). For the RHPLC analysis, we followed the procedures described by Guo et al. (1999). We used a low pressure gradient HPLC system with LC-10ATvp quaternary pump, UV detector (Shimadzu SPD-10ATvp, Macherey-Nagel, EC 250/4.6 Nucleodur 100-5 C18 column) and 20 μL loop and equipped with a Cd(Te) detector equipped with a RAD-501 single channel analyzer. The HPLC process was run using 0.1% TFA/acetonitrile, 0.1% TFA/water at a flow rate of 1 mL/min. The flow rate was set at 1 mL/min. The UV detector was settled at 240 and 280 nm.

2.5. Radiochemical synthesis of ^{99m}Tc-citro-folate

0.5 mg (0.83 μmol) of synthesize citro-folate was dissolved in 1 mL DMSO. The following solutions were sequentially added to a glass tube: 300 μL citro-folate, 100 μL tin chloride (1 mg $SnCl_2.2H_2O$/1 mL H_2O), 37 MBq ^{99m} Tc-sodium pertechnetate. The glass tube was incubated for 20 min. The radiochemical product was then analyzed by RTLC and RHPLC for purity. Briefly, 5 μL samples were spotted at the origin of two ITLC-SG strips (1.5 cm × 10 cm). The strips were developed in two different solvent systems (0.9% sodium chloride solution and acetone) and then scanned using the TLC scanner.

2.6. Radioelectrophoresis procedure

This process involved a Gelman electrophoresis chamber. Cathode and anode poles and application points on cellulose acetate strips were marked and moistened with 0.9% NaCl solution. Each compound was set on one strip and placed in the chamber. Standing time and applied voltage were set at 90 min and 250 V, respectively. The strips were then counted using the TLC Scanner.

2.7. Stability of ^{99m}Tc-citro-folate in human serum

In vitro stability of ^{99m}Tc-citro-folate was determined by incubating 300 μL of the labeled compound with 600 μL of human serum at 37 °C. The solution was then analyzed in time intervals of 15, 60, 180, 240 and 1440 min by radioelectrophoresis and RTLC.

2.8. Lipophilicity

The lipophilicity (log *P*) of the radiotracer was measured as follows: 100 μL of the radiolabeled conjugate, ^{99m}Tc-citro-folate, was added to a premixed suspension of 3 mL n-octanol in 3 mL water. The resulting solution was mixed for an hour at room temperature and allowed to stand for the formation of phases. Solution of 100 μL of each phase was removed and radioactivity was counted using a Cd(Te) detector. The experiments were conducted in triplicate and the measurements were averaged.

2.9. Biodistribution studies in organ tissues with saturated and unsaturated FRs

All animal experiments were approved by the Animal Ethics Committee of Ege University, and performed in accordance with the published guidelines. The saturated study was designed to establish reference measurements for comparison of the radioactivity obtained from the unsaturated study. The biodistributions of the radiolabeled conjugate in organs with saturated and unsaturated FRs were investigated using 18 female Albino Wistar rats with weight range between 100 and 150 g. The rats were randomly divided into 6 groups, each with 3 rats, for the FR saturated and unsaturated (control) studies at 3 different time points (60, 120 and 180 min). The biodistribution readings from the different organs of each rat were recorded and the results were group-averaged to obtain final measurements.

For the FR saturation study; each animal was administered folic acid, 300 μg (0.68 μmol) intraperitonally. After 30 min of folic acid administration, ^{99m}Tc-citro-folate was injected through the same route. The uptake efficacy of the radiolabeled conjugate was assessed at 60, 120 and 180 min post-injection to determine whether the uptake of the conjugate was specific in the receptor expressing target tissue. Lower radioactivity implied saturation of the FRs by the prior injected unlabeled folic acid and hence

interpreted as the indicator of the specificity of the radiolabeled conjugate. The percent of radioactivity per gram of tissue weight (%ID/g) was then calculated from the measurements.

For the receptor unsaturated study, we did not administer folic acid, but ^{99m}Tc-citro-folate conjugate (specific activity = 1.32 MBq/mmol), 20 μg (0.03 μmol), was injected only through the same intraperitoneal route to the rat. The animal was sacrificed and the organ tissues were again harvested at 60, 120 and 180 min of the injection. All tissues were weighed and counted for their radioactivities as has been done in the saturation study. The difference between the radioactivities of the sample organ from the saturated and unsaturated studies was used as the indicator of the binding efficiency of the manufactured conjugate in that target tissue.

2.10. Statistical analysis

Differences in the mean values of measured activities were evaluated statistically by SPSS 10 programme (Univariate Variance Analyses and Pearson Correlation). Probability values <0.05 were considered to be significant. A Pearson Correlation between the organs of saturated and unsaturated animals, testing for ^{99m}Tc-citro-folate was obtained.

3. Results and discussion

3.1. Characterization of citro-folate conjugate

The intermediate compounds (NHS-folate and folate-hydrazide) were in part synthesized during the process of manufacturing citro-folate were confirmed one-by-one by TLC and HPLC methods. *Rf* values from the TLC analysis of each compound were distinctly separate as listed in Table 1. HPLCs in Fig. 3 also indicated different retention times (*Rt*) for these compounds.

The products NHS-folate (N-hydroxysuccinimide-folate), folate-hydrazide and citro-folate were identified by LC–MS/MS and had molecular masses 527, 455 and 615, respectively. Characterization of citro-folate conjugate by ^{1}H NMR spectroscopy at 400 MHz yielded its structure ^{1}H NMR (DMSO): δ 2.05–1.82 (m, 2H, CH_2CH_2CH, 25), 2.41–2.15 (m, 2H, CCH_2CH_2, 26), 4.32–4.28 (q, 1H, CH_2NH, 24), 4.49 (d, J = 4.8 Hz, 2H, $NHCH_2CH$, 12), 6.63 (d, J = 9.2 Hz, 2H, $CHCHC$, 15–19), 6.91 (m, $CHNHC$, 1H, 23), 7.67 (d, J = 8.8 Hz, 2H, $CCHCH$, 16–18), 8.03 (m, $CNHCH_2$, 1H, 13), 8.64 (s, $CCHN$, 1H, 13). The spectrum was analyzed using the spectrometer's software. The reported chemical shifts are against TMS (tetramethylsilane). The proton spectroscopy has been proven to be the most valuable tool for determining the linkage property of azide group. Since, the azide group can coordinate with the main structure in two possiblities, γ or α position (Fig. 4). The spectrum of the compound folate-hydrazide in aromatic and aliphatic regions shows 9 resonance peaks. These well-resolved peaks correspond to five different aromatic ring protons, and the remaining aliphatic peaks are attributed to –CH and –NH groups in the chain. The position of azidation (γ or α) is identified with the assistance of CH proton at position 24 (Fig. 4). Azidation at α position shifts the peak at 24 upwards by about 5 ppm, when compared to the spectrum of folic acid. But, in our spectrum, no remarkable change was observed around this peak. Differently from folic acid, the spectrum of folate-hydrazide exhibited a peak at 4.33 ppm, which suggested azidation at γ position. γ position provides a binding site for folic acid to FR, and hence it is a desirable feature of a synthesized conjugate designed to target FR. In this regard, our compound folate-hydrazide shares this feature and plays a significant role in increasing the affinity of citro-folate towards FR. Collectively, the results from the different evaluation methods for defining molecular structure clearly demonstrated the successful synthesis of the citro-folate conjugate using our experimental protocols.

Table 1
Rf values of folic acid, NHS-folate, folate-hydrazide, citric acid and citro-folate compounds in 2-propanol:chloroform (7/3) solution.

Compounds	*Rf* values
Folic acid	0.2
NHS-folate	0.6
Folate-hydrazide	0.2
Citric acid	0.7
Citro-folate	0.8

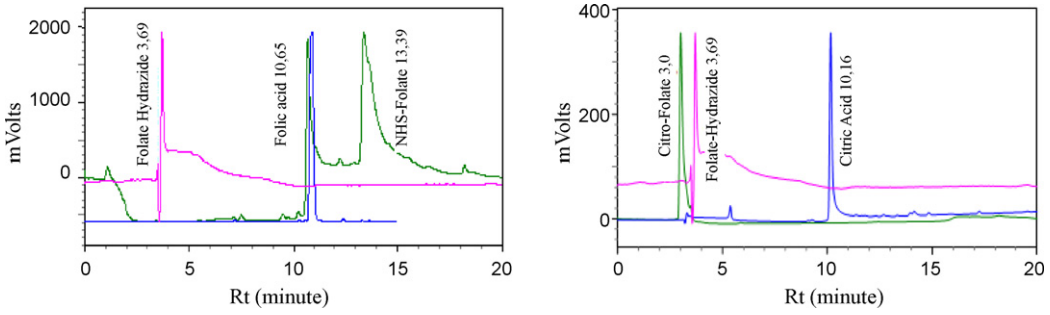

Fig. 3. HPLC chromatographies of folic acid, NHS-folate, folate-hydrazide, citric acid and citro-folate compounds.

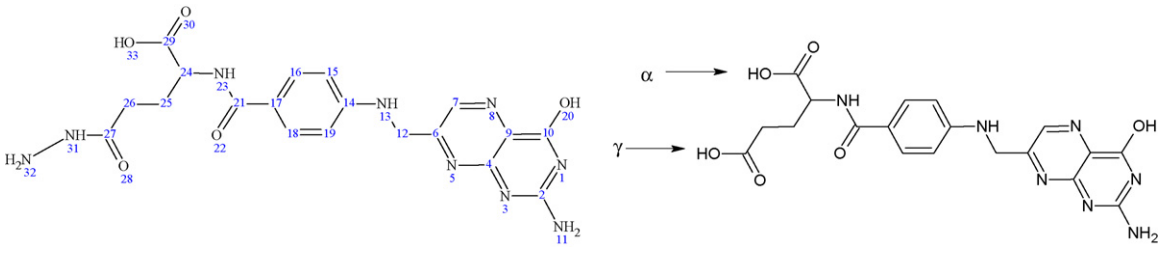

Fig. 4. Structure of folate-hydrazide and folic acid.

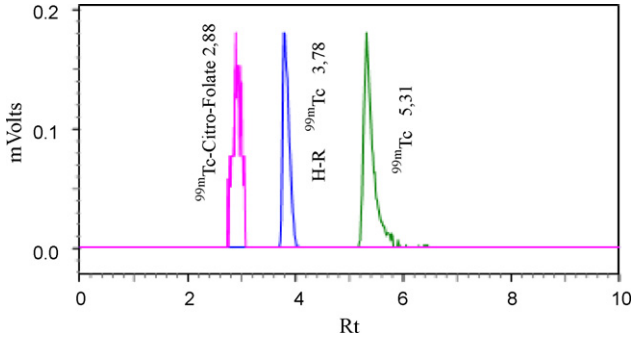

Fig. 5. RHPLC chromatograms of ^{99m}Tc-citro-folate, $^{99m}TcO_4^-$ and H-R ^{99m}Tc.

3.2. Quality control of ^{99m}Tc-citro-folate

The radiolabeling efficiency of the radiolabeled conjugate ^{99m}Tc-citro-folate was also confirmed using methods RTLC and RHPLC. The data obtained by examining the chromatograms showed that we were able to produce radiolabeled citro-folate with an efficiency of 97 ± 1.0%. The *Rf* values from RTLC for the other compounds ^{99m}Tc-citrat, ^{99m}Tc-citro-folate, $^{99m}TcO_4^-$, H-R ^{99m}Tc were 0.1, 0.03, 0.99 and 0.00, respectively, when acetone was used as the developing solution. But, when ACD was used as the solution, the corresponding measures were 0.97, 0.03, 0.96 and 1.00, respectively. In consistent with these results, *Rt* values from RHPLCs in Fig. 5 were 2.8, 3.78 and 5.31 min for ^{99m}Tc-citro-folate, reduced Tc-99m and pertechnetate, respectively. These results together provided the evidence that we were also successful in radiolabeling the conjugate of our interest. In vitro study on the stability of the radiolabeled conjugate demonstrated that human serum contained approximately 95.4% of ^{99m}Tc-citro-folate as an intact complex within 180 min after introducing it to the serum.

3.3. Biodistribution results

In vivo biodistribution studies involved experiments with rats in receptor saturated and unsaturated groups, and the results obtained from these studies were summarized in Figs. 6 and 7. According to the data in Fig. 6, the uptake of ^{99m}Tc-citro-folate in small intestine, large intestine, stomach, uterus and ovary was maximum at 60 min after the administration of the compound. The corresponding values were measured in the order of %ID/g: 0.10 ($p < 0.05$), 0.7, 0.07, 0.14 and 0.07, respectively. On the other hand, the uptake at 180 min decreased in the organs, [large intestine (%ID/g = 0.06 ($p < 0.05$)) and stomach (%ID/g = 0.02)]. The clearance of the radiolabeled folate conjugate was slower in the target organs equipped with FR than the non-targeted organs. While the readings in ovary and uterus were %ID/g 0.07 and 0.14 ($p < 0.05$) at 60 min, these uptake values decreased to %ID/g 0.04 ($p < 0.05$) and 0.02 at 180 min, respectively. These results together demonstrated that the uptake of ^{99m}Tc-citro-folate was FR mediated. This was further supported by the positive results obtained from the receptor saturated experiments as summarized in Fig. 7. According to the figure, the radiolabeled citro-folate uptake reached maxima at 60 min in the following organs: small intestine (%ID/g = 0.09), large intestine (%ID/g = 0.06) ($p < 0.05$), stomach (%ID/g = 0.09), kidney (%ID/g = 0.06), ovary (%ID/g = 0.06) and uterus (%ID/g = 0.07). In the order of organs presented, the corresponding %ID/g values at 180 min were all lower in magnitude: 0.04, 0.05, 0.03, 0.10, 0.02 and 0.02, respectively.

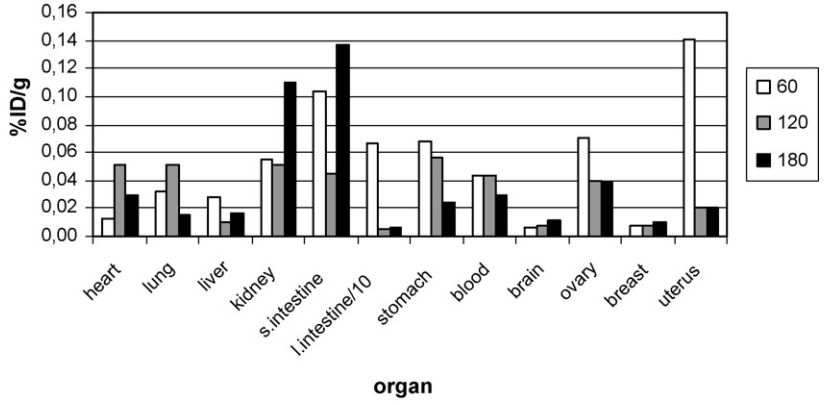

Fig. 6. Receptor unsaturated biodistrubition results of ^{99m}Tc-citro-folate conjugate in Albino Wistar rats.

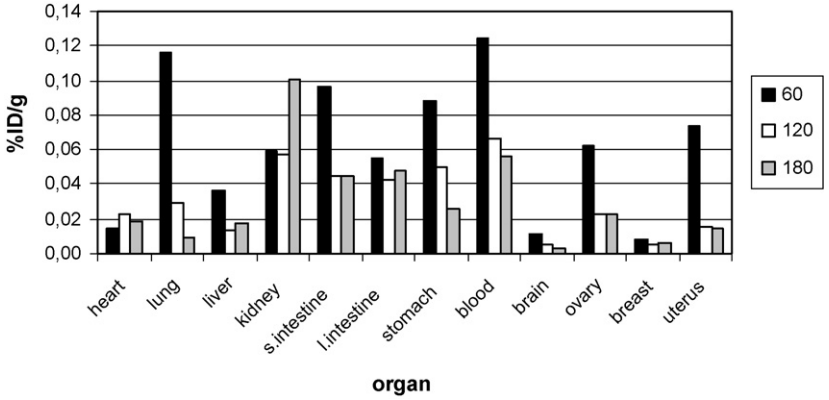

Fig. 7. Receptor saturated biodistrubition results of ^{99m}Tc-citro-folate conjugate in different organs at 60, 120 and 180 min after its injection.

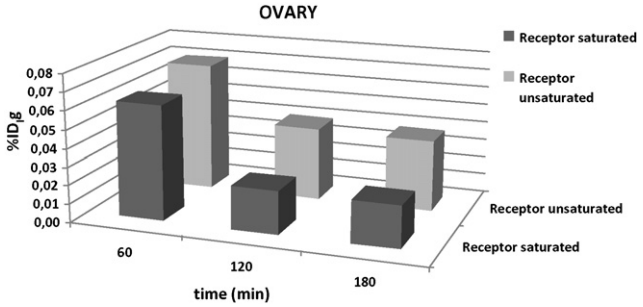

Fig. 8. Receptor saturated and unsaturated biodisturibition studies of ^{99m}Tc-citro-folate in ovary tissue.

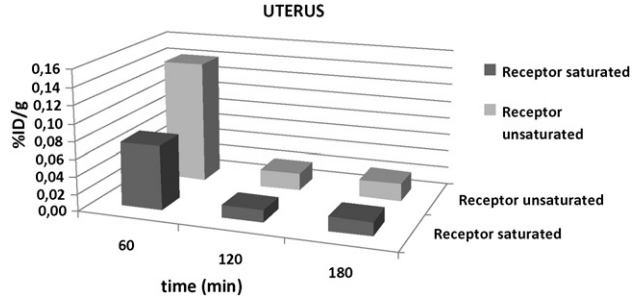

Fig. 9. Receptor saturated and unsaturated biodisturibition studies of ^{99m}Tc-citro-folate in uterus tissue.

The temporal uptake of ^{99m}Tc-citro-folate for ovary and uterus from the saturated and unsaturated receptor studies are depicted all together for side-by-side comparison in Figs. 8 and 9. Data in Fig. 8 shows that the uptake in ovary decreased when the receptor was saturated. The decrease was 11, 41 and 41% at 60, 120 and 180 min, respectively. The %ID/g values for uterus also decreased when the receptor was saturated as seen in Fig. 9. The uptake for this organ was 47, 31 and 26% at 60, 120 and 180 min, respectively.

The uptakes in uterus and ovarian were observed to decrease in saturated study (Figs. 8 and 9). Non-radiolabeled folic acid is able to reduce uptake in receptor rich tissues, such as the uterus and ovary. These results indicated that radiolabeled conjugate might be specific for ovary and uterus.

Our results indicated that the uptake is increased in kidney with time as shown in Fig. 6, suggesting shorter clearance time for the compound to be extracted from blood. To understand the origin of this behavior we performed additional studies to examine the lipophilicity of our conjugate. We used paper electrophoresis method to test the polarity of electric charge and also radiolabeling efficiency. The results from this test showed that both ^{99m}Tc-citric acid and ^{99m}Tc-citro-folate assumed negative charges. The lipophilicity analysis indicated low lipophility for the ^{99m}Tc-citro-folate conjugate. But, as shown in Table 2, the measured value was higher than the theoretical estimates calculated for folic acid or citro-folate. This difference may be attributed to the incompleteness of the assumptions that led to the theoretical derivations.

Table 2
Log *P* values of the compounds folic acid, citric acid, citro-folate and ^{99m}Tc-citro-folate.

Compound	Theoretical log *P*	Experimental log *P*
Folic acid	−2.32 ± 0.66	–
Citric acid	0.63 ± 0.40	–
Citro-folate	−2.35 ± 0.81	–
^{99m}Tc-citro-folate	–	−0.96 ± 0.39

Hydrophilic conjugates, such as ^{99m}Tc-citro-folate, remains longer in blood due to its negative charge. The increased circulation time leads to a rapid clearance of the conjugate from the blood by kidneys. These findings were consistent with the observations made with other hydrophilic compounds ^{99m}Tc-MAG3-MTX and ^{99m}Tc-MAG3-FA in the literature (Okarvi and Jammaz, 2006). In previous study performed with ^{99m}Tc-HYNIC-folate, Guo et al., suggested that hydrophilic radiopharmaceuticals exhibit special feature of fast clearance from blood pool through kidney. This property makes these radiopharmaceuticals ideal candidates for using in scintigraphic imaging (Guo et al., 1999). So far, several radiolabeled folate conjugates; ^{99m}Tc-PEG-folate (Kim et al., 2007) ^{67}Ga-DF-folate (Sudimack and Lee, 2000), ^{111}In-DTPA-folate (Wang et al., 1997), ^{99m}Tc-HYNIC-folate (Guo et al., 1999), ^{99m}Tc-EC-20 (Reddy et al., 2004), ^{18}F-FBA-folate (Bettio et al., 2006), etc. (has been synthesized using different chelation agents and evaluated for tumor imaging. But we labeled firstly citro-folate with ^{99m}Tc, and the efficiency of the radiolabeled conjugate was of 97 ± 1.0%, and evaluated in normal female rats.

Wang et al. (1997) indicated that the tumor to blood ratios of ^{111}In-DTPA-folate at 4 h post i.v. administration was 82 to 1. Siegel et al. (2003) confirmed that ^{111}In-DTPA-folate is safe, and possibly effective, for imaging of ovarian malignancy, perhaps allowing presurgical differentiation between malignant and benign ovarian masses. In performed study by Guo et al., the receptor binding property of ^{99m}Tc-HYNIC-folate was studied in cultured KB (a human oral cancer cell line) cells. It is shown that FR-mediated uptake was ~300 times the non-specific binding in the presence of 1 mM free folic acid (Guo et al., 1999). Sudimack and Lee (2000) demonstrated that ^{67}Ga-DF-folate was shown to have 100 times greater uptake in FR-positive oral carcinoma KB cells than the non-targeted ^{67}Ga-DF. On the other hand, in cell culture study performed by Okarvi and Jammaz (2006), it was proved more than affinity to FR ^{99m}Tc-MAG2-folate to ^{99m}Tc-MAG3-folate. In another study related to PEG-folate results show that the images were recorded at 30 min, 1, 2, 3 and 4 h after administration of ^{99m}Tc-PEG-folate. Until 4 h, the images especially showed specific binding to the tumor and strong uptake in comparison with normal muscle. Moreover, the tumor-to-normal muscle ratio reached 4.3 at 4 h. In the competition study, the tumor uptake was blocked by the co-injection of free folic acid, and the image indicates their conjugate is not taken up via the FR for tumor localization, and the tumor-to-normal muscle ratio was under 1.2 at 4 h (Kim et al., 2007).

Given these vast amounts of previous studies, our compound ^{99m}Tc-citro-folate is an incremental addition to the extensive library of folate conjugated radiopharmaceuticals and the radiolabeled conjugate showed localization in the tissues including folate receptor as uterus and ovary. The result demonstrated the ability of ^{99m}Tc-citro-folate in the express FRs tissues.

4. Conclusion

Our results in this study demonstrated that it is possible to successfully label citro-folate with ^{99m}Tc at high labeling efficiency (97 ± 1.0%) and the resulting radioactive conjugate ^{99m}Tc-citro-folate remains stable in serum and is feasible for in vivo use. The compound has greater affinity to those cells expressing FR as indicated by the accumulation of the compound at the FR-mediated organs such as uterus, ovarian and breast. The results of this study are significantly encouraging to bring about further evaluation of the radiolabeled folate conjugate as a possible folate receptor-positive tumors imaging agent.

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